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Imaging Mouse Development with Confocal Time-Lapse Microscopy

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Abstract

The gene expression, signaling, and cellular dynamics driving mouse embryo development have emerged through embryology and genetic studies. However, since mouse development is a temporally regulated three-dimensional process, any insight needs to be placed in this context of real-time visualization. Live imaging using genetically encoded fluorescent protein reporters is pushing the envelope of our understanding by uncovering unprecedented insights into mouse development and leading to the formulation of quantitative accurate models.

1. Introduction

The goal of developmental biology is to understand how embryos develop from simple fertilized eggs into complex animals of diverse shapes, and elaborate but stereotypical internal architectures. A carefully orchestrated series of events takes the zygote to the generation and organization of groups of cells with distinct developmental fates. As cells acquire distinct fates, they adopt specific behaviors that drive growth and shape changes. Thus, once specified, groups of cells will become organized into the tissue layers that go on to form the organs of the fetus and adult organism. Therefore, to accomplish embryonic development, cells must execute and coordinate a variety of diverse behaviors including proliferation, apoptosis, migration, and differentiation. Key to unraveling these events is the observation of embryos as they develop, by microscopic imaging. Today live imaging provides unbiased, quantitative, digitized data that can be used to formulate experimentally testable models of how biological circuits regulate developmental processes.

The mouse is currently the preferred genetically tractable mammalian model organism. Thus, experimentally investigating mouse embryonic development provides an important framework for understanding mammalian embryonic development. Mouse, and hence mammalian embryonic development, is a highly dynamic and complex process, which we are only beginning to understand with respect to the intrinsic molecular control and cellular dynamics. However, if we are to understand mouse development at the molecular and cellular level, a great deal more needs to be learned about the dynamics of gene expression, cell behaviors, and fate, and how they are molecularly determined and integrated. Most importantly, given the dynamic nature of embryonic development, any insights will ultimately need to be placed in a spatiotemporal framework which can only be achieved by live imaging.

Recent advances in live imaging and consequently our understanding of the cellular dynamics driving mouse embryo and tissue morphogenesis have been spearheaded by the convergence of four key technologies. These include: (1) the availability of mouse mutant strains in which a process of interest is defective, (2) the availability of mouse reporter strains for live imaging populations of cells or tissues of interest, (3) methods for culturing specimens (embryos or tissue explants) of interest for live imaging, and (4) improved modalities for image data acquisition and methods for data processing.

Transgenic and gene targeting techniques for random and directed mutagenesis of the mouse genome are routine, and offer the possibility to study gene function *in vivo*. Genetically modified mice often serve as excellent models of human congenital diseases. Over the past decade, forward genetic screens using chemical (e.g., ENU) or genetic agents (e.g., transposons) to induce random mutations into the genome have provided an additional unbiased source of mutants and helped advance our understanding of gene function during the morphogenetic processes.

Microscopy has always been an essential tool in developmental biology both for determining the normal course of events and for contrasting with the effects of experimental perturbations. Staining tissues or labeling specific groups of cells in tissues has extended the information content of microscopic observation. Various labeling methods have been used to deliver readily visualized reporters into cells in mouse embryos or organ explants, and have been widely used to study cellular dynamics and fate. Traditionally, a variety of approaches have been used to label single or groups of cells in mouse embryos. Embryological methods include grafting of genetically distinct cells or tissues (Kinder *et al.*, 1999, 2001), injection of vital dyes (e.g., DiI or DiO), or the electroporation of nucleic acids or proteins (Bronner-Fraser and Fraser, 1988; Haas *et al.*, 2001). Over the past two decades, transgenic methods have been used to express reporter genes in cells or tissues of interest. Additional temporal control of reporter expression is afforded by binary transgenic systems, such as the genetic-induced fate mapping (GIFM) approach (Joyner and Zervas, 2006; Nagy, 2000).

The GIFM method relies on characterized *cis*-regulatory elements to drive tissue-specific expression of a genetically encoded reporter. By contrast, many of the embryological techniques are invasive, or are only applicable when tissues are accessible for manipulation. As a consequence, cells which lie in deeper tissue layers of an embryo may be difficult to label. Furthermore, it still remains a challenge to label single cells or groups of cells, which can then be visualized at single-cell resolution, by many of these methods. Most importantly, however, the majority of these methods only provide a static picture of the highly dynamic three-dimensional processes of mouse development. In this way, dynamics are inferred through the analysis of multiple samples, as opposed to the reiterative analysis of a single sample, which is the goal of any live imaging experiment.

1.1. Genetically encoded reporters for live imaging cellular dynamics during mouse development

Researchers have long exploited molecular fluorescence to observe the localization and dynamics of proteins, organelles, and cells. The main classes of fluorophores in use today are small organic vital dyes (such as DiI or DiO), inorganic nanocrystals which are known as quantum dots (QDs), and genetically encoded fluorescent proteins (FPs) (Giepmans *et al.*, 2006). Even though the smaller size of vital dyes and QDs makes them attractive over FPs, they need to be conjugated to protein-targeting molecules, such as antibodies, which serve to restrict their application for live imaging experiments. By contrast, FPs can, in principle, be fused to any protein of interest making them minimally invasive and ideal reporters for live imaging.

Recent advances in the isolation and availability of genetically encoded FP reporters (Nowotschin *et al.*, 2009b; Shaner *et al.*, 2005) in combination with increasingly sophisticated imaging acquisition and analysis methodologies have propelled the field to investigate mouse development in living specimens and in three dimensions (3D) through live imaging (Hadjantonakis *et al.*, 2003). Besides cytosolic reporters represented by native FPs, subcellularly localized FP fusions such as fusions of FPs to human histone H2B that label active chromatin (Fraser *et al.*, 2005; Hadjantonakis and Papaioannou, 2004), or

fusions directed to the plasma membrane proteins by, for example, glycosylphosphatidylinositol (GPI) tagging (Larina *et al.*, 2009; Rhee *et al.*, 2006), facilitate the visualization of different aspects of a cell, and of individual cells in complex tissues. By segmenting nuclei, single cells can be identified and tracked in complex populations. Indeed, nuclei are easier to track than whole cells since they have a simple spherical morphology, are separated in 3D space, so making them easier to segment. Importantly, H2B fusions have a distinct advantage over standard nuclear localization sequences. They facilitate tracking of daughter cells, since they remain associated with chromatin during cell division (Nowotschin *et al.*, 2009b). By combining nuclear and membrane labeling yields, live information equivalent to routine histology, such that dynamic cell behaviors including cell division, death, or morphology, can be acquired and quantified at high resolution in living specimens. Cell-type-specific resolution can be achieved when expressing these FP reporters under defined *cis*-regulatory elements. For example, our laboratory has recently generated mouse strains that express GFP and RFP under visceral endoderm-specific *cis*-regulatory elements, from the mouse *Afp* (*Alpha-fetoprotein*) and *Ttr* (*Transthyretin*) loci, respectively, where all visceral endoderm cells are labeled with a green or red fluorescent reporter, respectively (Kwon and Hadjantonakis, 2009; Kwon *et al.*, 2006).

Recently, a powerful tool for highlighting specific pools of molecules has emerged with the engineering of photomodulatable FPs (PM-FPs). PM-FPs offer an increase in spatiotemporal resolution of labeling for visualizing dynamic cell behaviors and mapping cell fate. PM-FPs undergo a conformational change leading to a change in FP color when illuminated briefly with light of a certain wavelength that can be used to label and track subpopulations of cells or even single cells in embryos or tissue explants in a noninvasive and selective manner (Chudakov *et al.*, 2004; Gurskaya *et al.*, 2006; Lippincott-Schwartz and Patterson, 2008; Nowotschin and Hadjantonakis, 2009; Patterson, 2008; Stark and Kulesa, 2005, 2007; Wiedenmann *et al.*, 2004).

To distinguish between, and simultaneously image, different cell populations, spectrally distinct reporters can be used (Nowotschin *et al.*, 2009a). Multiplexing of reporters often necessitates microscope systems capable of spectral separation. Instruments with these capabilities are quite common and likely available at most institutions.

Live imaging of other organisms such as *Drosophila* (Murray and Saint, 2007), zebrafish (Scherz *et al.*, 2008), and *Xenopus* (Davidson *et al.*, 2008; Woolner *et al.*, 2009) has shown to deliver massive data sets about the dynamic relationship between cells during embryonic development. By contrast, the mouse embryo poses a significant challenge for live imaging due to its in utero development. The mouse embryo is not readily accessible for visualization and therefore needs to be cultured *in vitro*. Therefore, it is imperative, when live imaging embryos, to create *ex utero* culture conditions that most resemble *in utero* development and to use genetically encoded fluorescent reporters that exhibit a minimum brightness at the levels they are expressed in the embryo to be able to be detected with low laser power.

1.2. Live imaging mouse development

Among the most important technical advances in optical imaging is the capability for optical sectioning, which is routinely facilitated by the use of a confocal microscope. Confocal imaging allows the observer to look deep inside a sample without its physical destruction and by cutting out interference from out-of-focus light and scatter. Improvements and affordability in confocal instruments have partnered the development of increasingly sensitive detectors, leading to acquisition of data of unprecedented spatial and temporal resolution. In this chapter, we provide a detailed description of the methods and conditions that we routinely use for culturing and time-lapse imaging of mouse embryos. We discuss requirements for both pre- and postimplantation stages that are routinely used to obtain high-

resolution 3D time-lapse data revealing the gene expression, cellular and tissue dynamics of mouse embryonic development.

Once a reporter strain labeling cells of interest has been identified and imported from another investigator or a repository, or else generated within the lab, the steps of any live imaging experiment are quite simple. Animals are timed mated to produce embryos of defined stages. Then the actual imaging experiment can be divided into embryo dissection and mounting, followed by image data acquisition and analysis.

2. Materials

2.1. Media

Media for recovering and culturing embryos are commercially available from several vendors. Catalog numbers and vendor names are given for the media routinely used in our laboratory. However, alternative products will likely also suffice. In some cases, we also provide protocols for the homemade preparation of media compositions.

2.1.1. Media for recovering preimplantation embryos—M2: Commercially available from Millipore, Specialty Media (Cat. no. MR-015-D) or can be made up from constituent solutions (see Recipe for the preparation of 100ml of M2 on next page, and table of media compositions below).

2.1.2. Media for culturing preimplantation embryos—KSOM + amino acids: Commercially available by Millipore, Specialty Media (Cat. no. MR-121_D) or can be made up from constituent solutions (see Recipe for the preparation of 100ml of KSOM on next page, and table of media compositions below).

Important: KSOM should be stored frozen (−20 °C) for no more than 3 months, and once thawed, aliquoted and kept at (4 °C) for no more than 2 weeks.

Media compositions for preparing M2 and KSOM:

	Component	g/100 ml	Source	Cat. no.
Stock A (10×)	NaCl	5.534	Sigma	S3014
	KCl	0.356	Sigma	P9541
	KH ₂ PO ₄	0.162	VWR	BDH0268
	MgSO ₄ ·7H ₂ O	0.293	Sigma	M5921
	Sodium lactate 60% syrup	2.610 or 4.349 g	Fisher	S326-500
	Glucose	1.000	Sigma	G8270
	Penicillin G	0.060	Sigma	P4687
	Streptomycin	0.050	Sigma	S1277
Stock B (10×)	NaHCO ₃	2.101	VWR	BDH0280
	Phenol red	0.001	Sigma	P3532
Stock E (10×)	HEPES	5.958	Invitrogen	11344-041
	Phenol red	0.001	Sigma	P3532
Stock C (100×)	Sodium pyruvate	0.036	Sigma	P2256
Stock D (100×)	CaCl ₂ ·2H ₂ O	0.252	VWR	BDH0224
Stock F (10,000×)	Na ₂ EDTA·2H ₂ O	0.0372	Sigma	E5134

Stock G (100×)	Glutamine	200 mM	Invitrogen	25030-081
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Weigh solids into media bottles and add an appropriate quantity of water. If sodium lactate syrup is used (for Stock A), the weigh boat should be rinsed well and the wash added to the media flask. For Stock E, add half the required volume of water, then adjust the pH to 7.4 with 1 N NaOH. Make up to final volume using measuring cylinder. Filter all stock solutions through a 0.45 µm (Millipore) filter, aliquot into sterile tubes.

Preparation of 100 ml of M2 and 100 ml of KSOM from concentrated stocks:

Stock	M2 (ml)	KSOM (ml)
A (10×)	10.0	10.0
B (10×)	1.6	10.0
C (100×)	1.0	1.0
D (100×)	1.0	1.0
E (10×)	8.4	–
F (100×)	–	1
G (200×)	–	0.5
Water	78.0	76.5
BSA	400 mg	400 mg

BSA (bovine serum albumin), embryo tested (Sigma A3311).

Rinse all pipettes and tubes thoroughly into the final flask. When preparing BSA, allow to dissolve and gently swirl the medium without excessive frothing using a magnetic stirrer.

- **M2:** If necessary, readjust the pH to 7.2–7.4 with 1 N NaOH. Make up to final volume. Filter sterilize through a 0.45 µm (Millipore) filter and aliquot into polypropylene tubes. Store at + 4 °C.
- **KSOM:** Gas with 5% CO₂ in air to adjust pH to 7.4, alternatively incubate the medium for several hours (or overnight) at 37 °C, 5% CO₂ (in a tissue culture incubator) with the cap loosened. The pH will not usually require adjustment, but it may vary with different batches of BSA. Make up to volume. Filter sterilize and aliquot into polypropylene tubes.

2.1.3. Media for dissection of postimplantation embryos—We routinely use two alternative media compositions for embryo dissection. We find both equally good at preserving embryo viability during dissection.

- 95% DMEM/F12 (1:1) (Invitrogen, 11330-057) + 5% newborn calf serum (Lonza, 14-416F).
- Modified PB1 with 10% fetal calf serum (Papaioannou and West, 1981; Whittingham and Wales, 1969).

Media composition for preparing modified PB-1 medium with 10% fetal calf serum:

1. Make up stock solutions and mix the indicated volumes.

	g/100 ml	ml
NaCl	0.9	68.96
KCl	1.148	1.84
Na ₂ HPO ₄ ·12H ₂ O	5.5101	5.44
KH ₂ PO ₄	2.096	0.96
CaCl ₂ ·2H ₂ O	1.1617	0.88
MgCl ₂	3.131	0.32
Na-pyruvate	0.02 in stock NaCl	22.40

2. Add the solutions below to make up 104 ml of medium.

Penicillin/streptomycin (100×)	1 ml
Glucose	104 mg
Distilled water	1.16 ml
Phenol red (1%)	0.1 ml

3. Add heat-inactivated fetal calf serum (56 °C for 30 min) to a final concentration of 10%.
4. Filter sterilize and aliquot.

2.1.4. Media for culturing postimplantation embryos

- DMEM/F12 (1:1) with GLUTAMAX (Invitrogen, 10565-018), 1% penicillin/streptomycin (Invitrogen/Gibco, 15140) and rat serum.

The percentage of rat serum versus DMEM (Dulbecco's modified Eagles medium) varies depending upon the embryo stage. Commonly, we use 50% rat serum and 50% DMEM for E5.5–E8.25, 75% rat serum for stages E8.5–E9.5 and 100% for stages older than E9.5.

2.1.5. Rat serum—We routinely use rat serum that is specifically collected and available from commercial vendors such as Harlan Bioproducts (Cat. no. 4520). However, we find that the most consistent quality, but more labor intensive, is a homemade rat serum preparation, which we provide below.

2.1.5.1. Preparation of rat serum

1. Anesthetize adult male rat with ether (fume hood!) or isofluorane, according to institutional IACUC policies. Note that all procedures involving mice or rats should be written up in laboratory animal protocols, and be compliant with institutional policies and federal regulations.
2. Make a V-shaped incision into the skin and peritoneum of the lower abdomen of the anesthetized rat. Expose the dorsal aorta by pushing aside the internal organs.
3. Puncture the aorta using a beveled butterfly needle (Vacutainer blood collection set, BD Bioscience) and collect the blood by pressing the outlet needle into a vacutainer blood collection tube.
4. When the rat is completely exsanguinated, place blood on ice and euthanize rat using a guillotine or use the triple kill method.

5. Take all collected blood samples and centrifuge them for 20 min at 1300×*g*.
6. Remove and collect the supernatant (serum) into a new tube and discard the pellet.
7. To remove remaining debris, centrifuge again for 10 min at 1300×*g* and keep supernatant.
8. Heat inactivate the serum at 56 °C for 30 min.
9. Filter serum under sterile conditions using a 0.45 µm filter.
10. Make 1–2 ml aliquots of the serum, freeze, and store at –80 °C. Aliquots can be stored frozen for up to 1–2 years.

2.2. Equipment

2.2.1. Embryo culture

- Two pairs of watchmaker's forceps #5 (Roboz, RS-4978) and small surgical scissors (Roboz, RS-5910) for embryo dissection
- 3.5, 6, and 10 cm plastic Petri dishes (Falcon, 351001; 351007 and 351029)
- Organ culture dishes (Falcon, 353037)
- 3.5 cm glass-bottom dishes (MatTek, P35G-1.5-14-C) or LabTek coverslip bottom chambers (NUNC, 155360; 155379; 155382)
- CoverWell perfusion chamber gaskets, 9 mm diameter; 1.0 mm deep (Invitrogen/Molecular Probes, C18140) or 2.0 mm deep (Invitrogen/Molecular Probes, C18141)
- Mouth pipette (homemade) consistent of a mouthpiece (HPI Hospital Products Med. Tech., 1501P-B4036-2), latex tubing (latex 1/8 in. ID, 1/32 in. wall, Fisherbrand, 22362772), and very fine pulled glass Pasteur pipettes using a 1000 µl pipette tip connector
- Plastic transfer pipettes (Fisherbrand, 13-711-7M) to transfer embryos stages E7.5 and older
- Pulled Pasteur pipettes
- Suction holding pipette (optional; Eppendorf CellTram Air, 5176000.017)
- 1 ml syringe and 26-gauge needle (Becton Dickinson, 309623), 30-gauge needle with a blunt end (Becton Dickinson, 305106). Use sandpaper or sharpening stone for blunting the needle.
- Embryo tested light weight mineral oil (Sigma, M8410)
- Incubator providing a humid atmosphere and constant level of 5% CO₂
- Roller apparatus (BTC Engineering, Cambridge, UK)
- Industrial gas supply containing gas mixtures of 5% CO₂/95% O₂; 5% CO₂/20% O₂, or 5% CO₂/5% O₂
- Microscope with an environmental chamber to keep temperature and gas levels stable throughout the culture
- Human eyelashes, or cat whiskers, sterilized with 70% ethanol. (*Note:* To prevent animal cruelty, cats should not be harmed during whisker collection. We therefore recommend the use of cat whiskers which have been naturally shed by the animal.)

2.2.2. Microscopes

- Stereomicroscopes for dissecting and immobilizing embryos with both incident and transmitting light, and a magnification range from 6.3× up to 100×. Though not providing high-resolution information, these systems can also be used for time-lapse imaging to visualize changes in the gross morphology of embryos. Software controlling CCD cameras routinely used on stereo dissecting microscopes often allows time-lapse acquisition. To set up a culture for live imaging, a Petri dish can be covered with a watch glass or glass lid to prevent evaporation, and an incident air stream heater can be used for heating the sample. In such a system, media containing embryos is gassed intermittently.
- *Single point laser scanning confocal systems*: comparable to Zeiss LSM700 or 710. This is our system of choice for imaging mouse embryos in whole mount from preimplantation through to early somite stages or in explant at various stages.
- *Slit scanning confocal systems*: comparable to Zeiss LSM 5 LIVE systems. This type of system facilitates high-speed imaging, and can be used for visualizing blood flow, calcium dynamics, or the movement of organelles such as cilia.
- *Spinning disk laser confocal systems*: comparable to Perkin-Elmer UltraView RS5 systems. These systems work well for imaging preimplantation or very early postimplantation stage embryos, for reasonably high-speed imaging or for high-resolution cellular imaging. In our experience, spinning disk systems are excellent for imaging a single fluorophore, GFP or RFP in our experience, but when multiplexing, many systems are prone to substantial cross talk, especially when levels of reporter expression are low.
- *Two photon excitation systems*: can be used for live imaging of mouse embryos, but only offer benefits if deeper tissue imaging is required. TPLSM systems are usually more expensive to purchase and maintain. They often also require more time to set up imaging parameters in an experiment, and it is sometimes difficult to maximally excite multiple reporters due to the single tunable laser line present on most systems.
- For live imaging mouse embryos, we prefer to have the confocal system fitted onto an inverted microscope. When using systems available through an Institutional core facility, one may not have a choice of upright versus inverted systems. Though not optimal, we have also had some success with homemade chambers fitted onto the stage of upright microscopes. Two benefits of using an upright microscope are the ability to use water immersion (or dipping) objectives, that can be physically placed into culture media just above the specimen, and the ease of positioning or manipulating the sample. The shortcoming of an upright microscope-based system is the difficulty of maintaining a closed, gassed, humidified, and heated environment which is critical for successfully live imaging mouse embryos or tissues.
- The microscope and optics carrier are enclosed in an incubator providing a heated, humidified, and gassed environment for on-stage cell and embryo culture (Fig. 20.6). These are commercially available from microscope manufacturers (usually the most costly option), specialist manufacturers of incubators for microscopes, or can be made in-house (usually the least costly option). They can be fitted onto both upright and inverted microscopes, though the latter is our system of choice.
- The microscope should be equipped with a 5× dry objective to scan the field of view, and position the embryo. A 10× dry objective can be used to image low-magnification 3D time-lapses. A 20× dry or 25× multi-immersion, as well as a 40×

multi- or oil-immersion objectives, respectively, are used for high-magnification 3D time-lapses and acquisition of static images of fixed embryos. We rarely use 63× or higher magnification oil-immersion objectives for the acquisition of z-stacks of mouse embryos.

- Computer workstation running software for image data acquisition (Zeiss AIM—<http://www.zeiss.com/>, Perkin-Elmer Volocity—<http://cellular-imaging.com/products/Volocity/>, MetaMorph—<http://www.molecular-devices.com/pages/software/metamorph.html>) and image analysis (Amira—<http://www.amiravis.com/>, Volocity, Imaris—<http://www.bitplane.com/>, Metamorph).

3. Methods

3.1. Setting up timed matings for recovery of staged embryos

Mice are usually kept on a daily cycle of artificial light and darkness with the dark cycle starting usually at 6 pm and lasting 12 h. Mating is assumed to occur in the middle of the dark cycle. Breeding pairs or triangles of mice are set up using one male with one or two females, for pairs or triangles, respectively. It is preferable and usually more efficient for females that are in estrus to be preselected for matings. The morning after mating is set up, pregnant females are identified by the presence of a vaginal plug. By convention, females are aged as 0.5 days pregnant at midday of the next day. Embryos of the age of interest can be dissected accordingly. However, after dissection, embryos may be of the same age but are present as a variety of stages in any one litter, and so are usually staged by morphology, for example, using the nomenclature of Downs and Davies (1993) for early postimplantation embryos, or Theiler (1989).

3.2. Microscope setup and environmental control

Routinely, inverted microscope systems (Fig. 20.6) are used to live image cultured embryos. Unfortunately, it is not as easy to manipulate and position an embryo on an inverted system as it is on an upright one. The success of the *ex utero* culture of the embryo and the live imaging experiment depend on strict control of its environment that closely should resemble *in utero* development. Since mammalian embryos are very sensitive to variation in temperature, the culture condition should be able to accommodate a constant temperature of 37 °C. This can be achieved through an environmental chamber around the microscope stage (Fig. 20.6), which will keep the temperature and the gas content stable. Such a setup can either be built to personal specifications or commercially bought. The specific gas requirements vary for different embryonic stages. In general, the gas composition consists of 5% CO₂, a variable oxygen concentration and balanced nitrogen. Commonly, 95% O₂ is used for preimplantation embryos, 5% O₂ for early to mid postimplantation embryos (E6.5–E9.5), 20% O₂ for postimplantation embryos of stages E9.5–E10.5, 40% and 95% for late E10.5–11.5 (Nagy *et al.*, 2003). The gas is bubbled through a gas-washing bottle and directly supplied to the culture dish through tubing into a little plastic chamber placed on top of the dish.

Evaporation of the culture media needs to be kept to a minimum. For on-stage culture experiments, this is usually achieved by covering the culture dish with embryo-tested light mineral oil or, in cases when this is not possible, with water soaked paper towels placed around the culture dish. In roller culture experiments, using the intermittent gassing apparatus, tubes are sealed with silicone grease between periods of gassing.

When using an inverted confocal microscope system, embryos must be imaged through glass coverslips or dishes containing coverglass bottoms (e.g., MatTek dishes or LabTek 2-well glass coverslip chambers).

3.3. Culture and imaging of preimplantation mouse embryos

After fertilization, early mouse embryos float freely in the oviduct, which makes it easy to recover them from the mouse's oviduct. Culture conditions for *in vitro* culture of preimplantation mouse embryos have been very well established. Culture conditions should reflect the *in vivo* environment and therefore ask for appropriate media, temperature, and gas conditions. Preimplantation embryos are recovered in M2 medium and cultured in KSOM medium at 37 °C and 5% CO₂. The following section gives instruction on how to isolate, culture, and image preimplantation mouse embryos (Fig. 20.1).

1. Prewarm M2 medium and prepare and prewarm microdrop cultures of KSOM culture medium in a 35-mm culture dish covered with light mineral oil. The culture dish containing KSOM should be placed in a humidified incubator at 37 °C gassed with 5% CO₂ for at least 30 min for equilibration. The oil reduces the evaporation and pH and temperature changes when the dish is outside the incubator, though times of keeping embryos outside of proper atmospheric conditions should be minimized.
2. Sacrifice pregnant female by cervical dislocation. Make an incision into the lower abdomen and then dissect out:
 - the oviduct when looking for E0.5–E2.5 embryos. When dissecting out the oviduct, the distal part of the uterus should be left.
 - the entire uterus to obtain E3.5–E4.5 embryos.
3. Place oviduct or uterus in a drop of prewarmed M2 media.
4. Flush the oviduct with prewarmed M2 media using a 1 ml syringe with a blunt 30-gauge needle by inserting the needle in the infundibulum of the oviduct or the uterus with 26-gauge needle, respectively.
5. Collect the embryos using a mouth pipette attached to a pulled Pasteur pipette and transfer them through several microdrops of KSOM under oil to rinse off the M2 media completely. Then transfer them into the prepared culture dish containing prewarmed KSOM culture medium. *Note:* Transfer multiple embryos together since it has been shown that groups of embryos cultured in a small amount of medium increases blastocyst development (Lane and Gardner, 1992).
6. For live imaging place the preimplantation embryos in a glass-bottom dish with a drop of prewarmed KSOM covered with light mineral oil, equilibrated at 37 °C in a humidified incubator gassed with 5% CO₂ for at least 30 min. (*Note:* Prewarm the on-stage incubator to 37 °C before setting up the culture for imaging.)
7. Place dish on the microscope stage and provide gassing with 5% CO₂. Image embryos using either a single point confocal system or the spinning disk confocal system. *Note:* Minimize exposure to laser light to avoid phototoxic effect on cells of the embryo by using low laser power, minimal number of scans, increasing scan speed and the size of the optical section.

3.4. Culture of postimplantation mouse embryos for live imaging

As preimplantation embryos make their way down the oviduct to the uterus, they undergo several cell divisions and develop into blastocysts. The blastocysts hatch from the zona

pellucida and implant into the uterus, where they undergo the next steps of their development. Embryos that have implanted in the uterus are referred to as postimplantation embryos and are much more difficult to manipulate than preimplantation embryos. The following sections describe methods of dissecting, culturing, and imaging of postimplantation embryos (Fig. 20.1).

3.4.1. Collecting postimplantation embryos

1. Prewarm culture media in an organ culture dish by placing it into a humidified incubator at 37 °C and 5% CO₂ for at least 1 h before dissecting the embryos.
2. Sacrifice the female by cervical dislocation. Make an incision into the lower abdomen and then open up the peritoneum to remove the two horns of the uterus with the embryos and place them in a dish with dissecting media (DMEM/F12 + 5% NCS).
3. Dissect the decidua out from the uterus under a stereomicroscope. Then carefully remove the decidua and subsequent Reichert's membrane from the embryo using watchmaker's forceps (Fig. 20.2). *Important:* Dissection of embryos should be done in a timely manner – speed is of the essence! The yolk sac and the ectoplacental cone (EPC) of the embryo must not be damaged during dissection. Defective embryos will not develop properly in culture and should not be used for time-lapse imaging.
4. After dissection, immediately move the embryos using a transfer pipette into a dish with prewarmed culture media and incubate them in an incubator at 37 °C and 5% CO₂ for 15–20 min before setting them for on-stage culture (see also Section 3.4.3) or when doing roller culture immediately transfer embryos in prewarmed culture media in roller culture bottles (see also Section 3.4.2).

3.4.2. Roller culture of postimplantation mouse embryos—The most preferred culture method for mouse embryos, when not live imaged, is a roller culture system. Constant motion of the embryos in rotating bottles/tubes, together with the constant temperature and gassing provide most optimal *ex utero* culture conditions. Two alternative roller culture system designs are commonly used. One uses intermittent gassing of the culture, whereas the other one provides gassing throughout the culture period (Fig. 20.3). Both types of system are commercially available from BTC Engineering. Our laboratory has experience with both systems; however, the latter has become our preferred method for culturing embryos since continuous gassing and a constant level of humidity provides a homogenous environment for the duration of the experiment, and is most favorable for the normal development of embryos.

3.4.2.1. Roller culture with intermittent gassing

1. Prewarm the incubator and the culture media to 37 °C.
2. Culture media must be equilibrated with the appropriate gas combination at least 1 h before setting up the culture.
3. Dissect embryos as described in Section 3.4.1.
4. Transfer embryos into a tube with the appropriate culture media using 1 ml media per 1 embryo, seal tube tightly, and place them on the rotator that consists of several horizontal rollers.
5. Gas the culture media every 6 h with the appropriate gas combination for the stage of the embryo until the end of the culture.

3.4.2.2. Roller culture with constant gassing

1. Once you have prepared culture media and dissected the embryos, you are ready to set them up in the roller culture incubator.
2. Transfer dissected embryos into glass bottles, seal glass bottle with a silicone rubber stopper that has an opening to provide a constant supply of gas.
3. Attach glass bottles to the gassing system, a gas filled drum rotating around a horizontal axis. *Note:* Glass bottles should be rinsed with water or 70% ethanol and/or sterilized after each use.

3.4.3. Static culture (on-stage culture) and imaging of postimplantation mouse embryos

—Static or on-stage culture, though less ideal for the *ex utero* development of the embryo, is the preferred method to culture embryos for time-lapse imaging of postimplantation mouse embryos. Embryos can be cultured up to 18–36 h on-stage. (The mentioned time span does not only depend on the culture conditions but also very much on the intensity and frequency of laser light the embryos are exposed to!)

1. Before setting up the embryo for a time-lapse experiment, prewarm the on-stage incubator at the microscope to 37 °C.
2. Dissect embryos as described before and incubate them for 15 min at least in prewarmed and pregassed culture media.
3. Prepare glass-bottom dish (MatTek, P35GC-1.5-14-C) for the culture and imaging of the embryo for stages E5.5–E8.5. Place a drop of prewarmed culture media to fill up the glass part of the dish and cover it with embryo tested light mineral oil. If embryo needs to be immobilized during culture, modifications according to the stage of the embryo are applied to the glass-bottom dish. See Section 3.4.4 for a detailed description.
4. Set up the culture dish with the embryo on the microscope stage. Gas the embryo by putting a small square plastic cover with an outlet for 5% CO₂ creating an appropriate culture atmosphere.
5. Set up imaging experiment. Note that the success of the culture and the imaging experiment depends on a good balance between exposure to laser power and brightness of the image. In general, laser intensity and frequency of scans should be kept as low as possible, otherwise laser light will be phototoxic to embryonic cells. Scan speed and adjustment of the thickness of optical sections can also minimize laser light exposure. However, this is very much dependent on the brightness of the fluorophore used, microscope setup used, and the biological question asked. The optimal setup has to be established empirically using wild-type embryos for each microscope system.

3.4.4. Methods for immobilizing postimplantation mouse embryos for time-lapse imaging

—Immobilization of postimplantation embryos is necessary when a specific region of the embryo needs to be imaged repetitively. Various possibilities exist for immobilization of embryos. Suction pipettes, for example, can be used to hold the embryo while it is suspended in the culture. However, our preferred method of immobilizing embryos between E7.5 and E8.25 stages is to use modified culture dishes with inserts for mounting, for example, CoverWell chamber gaskets. These ‘press-to-seal’ gaskets are made of a plastic surface containing a hole (for inserting the embryo) and a rubber section that can be stuck onto the coverglass bottom of the culture dish. These gaskets are manually cut to fit the glass bottom of the imaging dish (Fig. 20.4). Different heights of these chamber gaskets are available to accommodate embryos of different sizes. Human eyelashes or cat whiskers

inserted through the EPC of the embryo are used to suspend the embryo through the plastic hole and fix the embryo's position (Fig. 20.5A–C). For E8.5 embryos or older, we use a modified culture dish with a miniature crane that will hold the embryo during on-stage culture (Fig. 20.5D and E). The embryo crane consists of a thin platinum wire extending from a cantilever, which is mounted on top of a one-coil spring. The spring is fixed onto a washer sitting inside the Petri dish, and is regulated in height by an adjustment screw coupled to the washer. The wire from the cantilever extends down over the middle of the Petri dish and into a medium-filled ultra high molecular weight polyethylene ring that is fixed in the center of the dish. The bottom tip of the wire is formed into a hook from which the embryo hangs suspended in the medium, and over the coverslip. The latter two methods are described below in greater detail.

3.4.4.1. Embryo immobilization using CoverWell chamber gaskets

1. Cut chamber gaskets to fit the glass bottom of your culture dish and adhere the rubber part to the bottom. To facilitate adhesion to the glass, place dish on a hot surface (i.e., heating block).
2. Add prewarmed and equilibrated DMEM/F12 culture media to the dish covering the entire gasket and cover it with light mineral oil.
3. Transfer embryo and eyelash/whisker to the dish.
4. Under a stereo dissecting microscope, pierce the EPC of the embryo and position the embryo in such a way that it hangs through the plastic hole of the dish and the eyelash/whisker balances the embryo at the top of the plastic (Fig. 20.5C).
5. Place dish on the microscope stage with environmental chamber using conditions for embryo culture described previously.
6. Note that the orientation of the embryo depends on the precise angle at which the eyelash/whisker is inserted into the EPC. If the embryo is not in the correct orientation, one can usually carefully remove and reinsert the eyelash/whisker. However, this process cannot be performed repeatedly until the desired orientation is achieved.
7. Image embryo (Section 3.5).

3.4.4.2. Embryo immobilization using an embryo “crane”

1. Before setting up the embryo for a time-lapse experiment prewarm the crane to 37 °C by placing in a tissue culture or on-stage incubator.
2. Dissect embryos as described previously and incubate them for at least 15 min in prewarmed and pregassed culture media in a tissue culture incubator.
3. Add prewarmed and equilibrated DMEM/F12 culture media to the inner ring.
4. Transfer embryo to the ring.
5. Using watchmaker's forceps, hang the embryo from the hook by piercing the EPC of the embryo and position the embryo (Fig. 20.5F).
6. Place dish on the microscope stage with an environmental chamber using the conditions for embryo culture described previously.
7. Image embryo (Section 3.5).

3.5. Live imaging cultured mouse embryos

While imaging, the frequency of acquisition, as well as the laser power used, should be kept to a minimum since laser light is phototoxic to cells and can impair proper development. Control cultures without exposure to laser light should be set up to work out the proper imaging conditions, especially when morphogenetic events in mutant embryos should be imaged.

3.5.1. Setting up the image data acquisition

1. Prewarm the microscope system to 37 °C for 2 h prior to starting the experiment. In the meantime the embryos are dissected, placed in a tissue culture incubator and then mounted. Of note, we prefer an incubator system (Fig. 20.6) that encloses the optics carrier and microscope stage over an on-stage incubator system. The former system is preferable as it provides more robust temperature control with fewer fluctuations, so preferable for sustained normal embryonic development, as well as preventing focal drift created by thermal expansion or contraction of the system's mechanical components.
2. Place coverglass-bottomed culture dishes containing immobilized embryo onto the stage insert so that it is securely in place. Since in many systems gassing occurs within the chamber above the stage, a secure fit of the dish within the stage insert is desired so as to limit gas leakage.
3. Find the embryo using a low-magnification (5×) scanning objective. This is usually done by visualizing the specimen in bright field and manually moving the stage using the x - y control.
4. Sequentially move through a series of dry objectives of increasing magnification (10× and 20×) refocusing and repositioning the stage to visualize the region of the embryo that you wish to image. We routinely use the 10× objective for low-magnification 3D time-lapse data acquisition to visualize global cell rearrangements (Fig. 20.7), and the 20× for higher magnification data acquisition where our aim is to be able to track individual cells. Occasionally, we will imaging using an immersion objective (25× or 40×), if so we add the oil, glycerol, or water-based immersion liquid onto the objective, move it out of position temporarily as we place the dish on the stage and scan it to identify the embryo, and then bring it back into position. Of note, since it readily evaporates, water cannot be used as an immersion medium for time-lapse imaging of embryos cultured at 37 °C. To overcome this issue, many water-based immersion compounds are available for use as substitutes.
5. Through the microscope software, set up the image acquisition parameters. The laser settings will vary based on the microscope system, and also on the reporter being visualized. Quite some effort needs to be invested in empirically evaluating each reporter strain to determine the optimal parameters for visualization without photobleaching of the reporter or phototoxicity to the sample. For example, we routinely use a Zeiss LSM 510 system with the following 25 mW 488 nm Argon laser (for GFP) output: running at 6.1 A with power set to <5% but preferably 2–3%. To increase the speed of data acquisition on a laser point scanning confocal we will routinely bidirectionally scan in 'single track' mode, where all channels are acquired at once. The alternative mode of 'multitracking,' where all channels are captured separately, may need to be used with overlapping fluorophores, but results in extended exposure of the specimen to laser light.

6. Through the microscope software, set the top and bottom limits of the *z*-stack as well as the *z*-spacing. When inputting these settings, make sure you account for inherent focal drift, embryo movement as well as slight drift and embryo growth by extending the *z*-stack by several micrometers at either end. We routinely acquire *z*-stacks of up to 150 μm with a spacing of 0.5–6 μm depending on the objective and experiment. For example, a 0.5 μm *z*-interval is not necessary when using a 10 $\times$ objective, which inherently produces optical sections of several micrometers.
7. Through the microscope software, set the time interval and the number of *z*-stacks that will be acquired in, or total duration of, the experiment.
8. Click START.
9. Once a 3D time-lapse is started, we routinely return to the microscope to check on the data as it is being acquired and to make sure that the embryo is not drifting. If we do find embryo drift, we stop and save any useable *z*-stacks. We then reposition the embryo, reset the top and bottom limits of the *z*-stack, and restart time-lapse acquisition. Furthermore, if the experiment is taking place during the day, one can always return to the microscope and readjust any settings, for example, the positioning in *x/y/z* for embryo drift, growth or focal drift of the system, laser power or gain for reporter photobleaching, degradation or changes in the level of expression.
10. At the end of the experiment, save the data and score the embryo, usually by placing onto a stereo dissecting microscope, for normal development.
11. Once the experiment is terminated, clean the objective used to image, even if it was a dry one. Then move the objective turret to an empty position or a position with a low magnification (5 $\times$), long working distance objective.
12. If imaging embryos are suspended with the ‘crane,’ clean the ‘crane’ by wiping it with 70% ethanol.

3.5.2. Choosing reporters

- An increasing number of FP reporter strains are available from public repositories such as the JAX IMR (<http://www.jax.org/imr/index.html>).
- It should be borne in mind that any particular reporter strains demonstrated to be readily detectable on someone else’s system may not necessarily work on every system.
- Fusing a protein of interest to an FP can affect its native behavior, and can in some cases subtly or significantly affect a developmental process being studied. Our test for developmental neutrality of any fusion that we generate is to determine if we can establish strains of transgenic mice exhibiting widespread expression of the fusion protein. Proof of developmental neutrality comes if germline transmission and expression of the transgene is achieved, transgenics are represented in Mendelian ratios, and are viable, fertile, and indistinguishable from nontransgenic littermates.
- A knowledge of the physical and optical properties of FPs is crucial to determine if and to what extent FPs might affect the localization, function, and spatiotemporal dynamics of fusion proteins. *Aequorea victoria* GFP variants have been shown to weakly dimerize. By contrast, FPs from *Anthozoa* species form obligate tetramers, a feature that precluded the use of first generation native proteins in mouse reporter strains, and has hampered the use of even newer generation variants in many fusion proteins. So although oligomerization does not preclude the use of newer

generation cytosolic FPs as reporters for gene expression, it cautions their use in fusions. In our experience, even though both human histone H2B–GFP and H2B–mCherry FP fusions are bright and localize to active chromatin, we have been unable to generate a strain of mice exhibiting widespread expression of an H2B–mCherry fusion. By contrast, we have developed several strains expressing the H2B–GFP fusion (Nowotschin *et al.*, 2009b). The brightness of an FP (the product of its extinction coefficient and its fluorescence quantum yield) determines the intensity of the fluorescence signal that can be visualized, and thus captured. Bright FPs require low-intensity illumination or short scan times for acquisition, both of which are preferable for live imaging where they serve to minimize phototoxicity and photodamage to the tissue, as well as to reduce photobleaching (excitation-induced photodestruction) of the fluorophore. We therefore recommend that the latest generation FP variants be used in the generation of genetically modified mouse strains. A minimal investment of time at the start of an experiment, to generate transgenic or gene targeting DNA constructs incorporating newer FP variants, is later rewarded when strains are available for imaging. Even though improved FP variants are continually being developed which are optimized for faster folding and chromophore maturation, increased brightness and photostability and minimal self-association, in our experience not all mouse transgenic strains will express reporters at robust enough levels for live imaging. Unfortunately, little can be done to improve the fluorescent signal in a reporter strain exhibiting low levels of expression.

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Figure 20.1. Overview of mouse embryonic development to midgestation. Preimplantation and early postimplantation stage mouse embryos are depicted. Stage-specific dissection, culture media, and gas composition are provided for each particular stage. Embryo images are not shown to scale. NCS, newborn calf serum; RS, rat serum; E, embryonic day.

Figure 20.2.

The dissection of postimplantation embryos for *ex utero* culture. Images show the sequence of dissection of a postimplantation embryo at stage E8.5: (A1) A deciduum, removed from the uterus, containing an embryo. (A2–A5) Panels show the careful, sequential peeling away of the decidual tissue (with watchmaker's forceps) leaving the yolk sac, which surrounds the embryo, intact. (A6) A dissected E8.5 embryo enclosed in its yolk sac. Note, small portion of ectoplacental cone (EPC) remaining on the top of the embryo. EPC should be left on to prevent rupturing of the yolk sac and for facilitating the immobilization of the embryo for culture. (B–D) Embryos of various stages dissected as shown in A1–A5: (B) E6.5 embryo, (C) E7.5 embryo, (D) E9.5 embryo.

Figure 20.3.

Two roller systems used for *ex utero* culture of mouse embryos. (A–C) Equipment for roller culture of mouse embryos with intermittent gassing: (A) incubator with roller apparatus inside, (B) close-up view of the roller apparatus, (C) tube for culturing embryos. For culture tube will be sealed with silicone grease. (D–F) Equipment for roller culture of mouse embryos with constant gassing: (D) incubator with drum inside, (E) close-up of drum, (F) bottle for culturing embryos. Bottle will be attached to drum and is connected to the gas flow inside the drum through opening on the top.

Figure 20.4.

Preparation of gasket chambers for immobilizing postimplantation embryos for static culture and time-lapse imaging. (A–E) Preparation of inserts for immobilization of early postimplantation stages: (A) CoverWell (Invitrogen, USA) gasket chambers; (B, C) chambers are cut so to obtain a plastic insert containing one hole for the insertion of the embryo; (D) pieces of plastic are cut away so to fit insert into an imaging dish; (E) adhesive side of the insert is stuck to bottom of the glass-bottom imaging dish.

Figure 20.5.

Two alternative methods for immobilization of postimplantation mouse embryos for static culture and time-lapse imaging. (A–C) Immobilizing a postimplantation embryo using a CoverWell gasket chamber: (A) glass-bottom imaging dish with a CoverWell gasket chamber adhered to the glass bottom; (B, C) close-ups of embryo after culture hanging through the hole in the plastic via an eyelash. (D–F) Immobilizing a postimplantation embryo using an embryo “crane”: (D) Embryo “crane,” made of stainless steel, inserted into the glass bottom of the imaging dish. A plastic ring is inserted into the middle of the dish above the glass bottom that holds the culture medium, where the embryo is hung into via a platinum wire extending from a cantilever. (E, F) Close-up view of an embryo after culture hanging from the platinum wire of the embryo “crane.”

Figure 20.6.

Enclosed incubator containing microscope systems for live imaging static cultured mouse embryos. (A) Single point laser scanning confocal inverted microscope with scan head placed on a base port. This arrangement facilitates unobstructed access to the microscope and placement of an environmental chamber enclosing the stage, objectives, and condenser. The microscope is positioned on an air table to buffer vibration and movement to the specimen during imaging. (B) Spinning disk confocal on an inverted microscope with laser module and Nipkow disk scan head on the left side of the microscope and custom-made environmental chamber. The microscope is positioned on a table top antivibration platform to buffer movement to the specimen during imaging.

Figure 20.7.

Live imaging of postimplantation mouse development, visualization of mesoderm cell dynamics during foregut invagination. Sequence of stills from a laser scanning confocal time-lapse experiment of an embryo expressing a histone H2B–YFP reporter in all mesoderm cells. Normal morphogenesis of the headfolds (hf) and invagination of the foregut (fg) are evident in the bright field panels of the time series. Embryo was imaged for a total of 8.5 h. Optical sections were taken every 4 μm , and z-stacks were acquired at 20 min intervals. Upper row shows a time series of bright field images. Middle row shows 3D rendered z-stack of the GFP channel from individual time points of the same time series. Bottom row shows the overlay of the GFP channel onto the bright field images. m, midline.